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REVIEW

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Vitamin B_{12}, folate, and the methionine remethylation cycle—biochemistry, pathways, and regulation

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Abstract

Vitamin B_{12} (cobalamin, Cbl) is a nutrient essential to human health. Due to its complex structure and dual cofactor forms, Cbl undergoes a complicated series of absorptive and processing steps before serving as cofactor for the enzymes methylmalonyl-CoA mutase and methionine synthase. Methylmalonyl-CoA mutase is required for the catabolism of certain (branched-chain) amino acids into an anaplerotic substrate in the mitochondrion, and dysfunction of the enzyme itself or in production of its cofactor adenosyl-Cbl result in an inability to successfully undergo protein catabolism with concomitant mitochondrial energy disruption. Methionine synthase catalyzes the methyl-Cbl dependent (re)methylation of homocysteine to methionine within the methionine cycle; a reaction required to produce this essential amino acid and generate S-adenosylmethionine, the most important cellular methyl-donor. Disruption of methionine synthase has wide-ranging implications for all methylation-dependent reactions, including epigenetic modification, but also for the intracellular folate pathway, since methionine synthase uses 5-methyltetrahydrofolate as a one-carbon donor. Folate-bound one-carbon units are also required for deoxythymidine monophosphate and de novo purine synthesis; therefore, the flow of single carbon units to each of these pathways must be regulated based on cellular needs. This review provides an overview on Cbl metabolism with a brief description of absorption and intracellular metabolic pathways. It also provides a description of folate-mediated one-carbon metabolism and its intersection with Cbl at the methionine cycle. Finally, a summary of recent advances in understanding of how both pathways are regulated is presented.

KEYWORDS

folate, hyperhomocysteinemia, methionine cycle, methylmalonic acidemia, one-carbon metabolism, vitamin B_{12}

1 | INTRODUCTION

Functional metabolism of vitamin B_{12} and folate is critical to human health. Vitamin B_{12} (cobalamin, Cbl) is not produced in humans but is required for the function of two enzymes, cytosolic methionine synthase (MS, EC 2.1.1.13) and mitochondrial methylmalonyl-CoA mutase (MUT, EC 5.4.99.2).[1] MUT utilizes the adenosylated form of Cbl to catalyse the conversion of L-methylmalonyl-CoA to succinyl-CoA. This is an essential step in the catabolism of branched-chain amino

acids, odd-chain fatty acids, and the side chain of cholesterol, and contributes to anaplerotic replenishment of the tricarboxylic acid cycle.[2] By contrast, MS requires the methylated form of Cbl and catalyzes the remethylation of homocysteine to methionine using 5-methyltetrahydrofolate as methyl donor. The importance of this reaction extends beyond the production of methionine, an essential amino acid, because methionine is further converted to S-adenosylmethionine (AdoMet, often called SAM). The methyl group of AdoMet can be donated to form a wide range of vitally important methylated compounds, for example, creatine, epinephrine, and sarcosine, as well as methylated DNA, RNA, and proteins. The utilization of 5-methyltetrahydrofolate as methyl donor by MS inexorably links Cbl metabolism with folate-mediated one-carbon metabolism, which in addition to the production of methionine and AdoMet, is required for de novo purine synthesis, production of deoxythymidine monophosphate (dTMP), and is an important cellular energy source through reduction of $NADP^+$ to NADPH. The importance of these pathways to human health is underlined by the extent and severity of disease caused by their primary and secondary dysfunction.

This review discusses Cbl and folate metabolism, with an emphasis on the intracellular pathways involved in Cbl cofactor synthesis, methionine remethylation, and one-carbon metabolism. The biochemical functions and regulation of individual proteins within these pathways are discussed, along with the biochemical/cellular consequences of their dysfunction. It is intended that this paper functions as a stand-alone summary of current knowledge of cellular Cbl and folate metabolism, while additionally may serve as a primer for those readers interested in the clinical manifestations of blocks in these pathways, presented in the adjoining paper "The clinical presentation of cobalamin-related disorders: from acquired deficiencies to inborn errors of absorption and intracellular pathways."

2 | COBALAMIN METABOLISM—STRUCTURE AND INTAKE

Once called "nature's most beautiful cofactor,"[3] Cbl is at least nature's most chemically complex coenzyme (Figure 1). The focal point of Cbl is the central cobalt atom, which can form between four and six bonds, and can exist in the reduction states cob(III) (most oxidized), cob(II), or cob(I)alamin (most reduced). There is a correlation between the cobalt oxidation state and its preferred number of coordinated elements, whereby cob(III)alamin often forms six bonds, cob(II)alamin forms five bonds, and cob(I)alamin forms four bonds. Of these, four cobalt bonds are always occupied by the nitrogen atoms of the planar corrin ring surrounding the cobalt. Cobalt may additionally bind a lower axial ligand, the dimethylbenzimidazole (DMB) moiety which attaches back to the corrin ring. When bound, Cbl is considered "base-on," when not "base-off." Finally, the cobalt atom may also or instead bind an upper axial ligand (R-group), which may consist of any number of compounds, but whose human cofactor forms include the adenosyl- (adenosylcobalamin, AdoCbl) or methyl- (methylcobalamin, MeCbl) moeities, while glutathionyl- (glutathionylcobalamin, GNCbl) and hyrdoxo- (hydroxocobalamin, OHCbl) are also physiologically important and cyano- (cyanocobalamin, CNCbl) is common in pharmaceutical preparations.

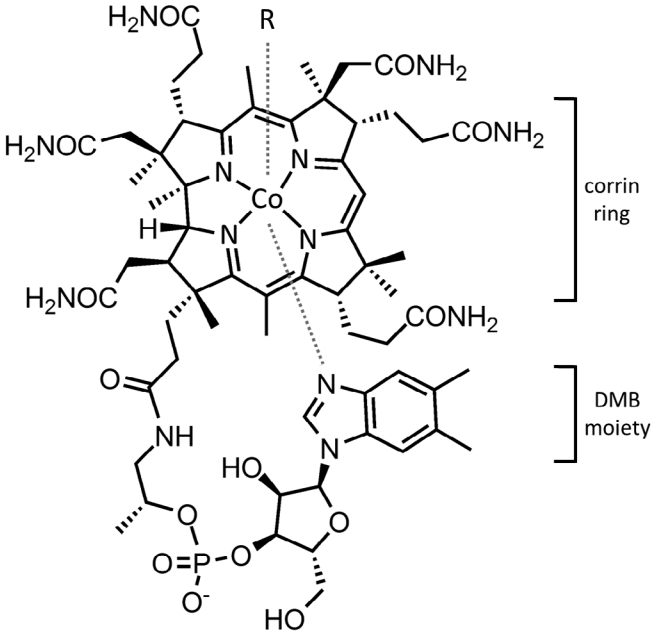

FIGURE 1 Chemical structure of vitamin B_{12} (cobalamin). The corrin ring and the dimethylbenzimidazole (DMB) moiety in the base-on configuration are indicated. Grey dashed lines represent nonessential bonds. R represents various upper axial ligands, including: adenosyl, methyl, glutathionyl, hydroxo, and cyano

Cbl approximates 1300 to 1500 Da in size, and its complete synthesis requires more than 25 different steps, which can occur aerobically or anaerobically[4] at large energetic cost. Complete Cbl synthesis is limited to selected Eubacteria and Archaea, whereas other Cbl-utilizing organisms must modify Cbl acquired following uptake from other sources.[5] In humans, these sources are limited to animal products, and therefore a certain proportion of the population, with low intake of animal products, is moderately vitamin B_{12} deficient,[6] even though dietary requirements are only a few micrograms a day.[1] Even processing of pre-synthesized Cbl is complicated and metabolically costly; currently, approximately 20 human genes are known to be involved in absorption, selection, transport, modification, and utilization of Cbl acquired from the diet.

Following ingestion of animal products containing vitamin B_{12}, rich sources of which include raw liver, kidney, shellfish, meat, or dairy products (United States Department of Agriculture (USDA) food composition database: https://ndb.nal.usda.gov/ndb/nutrients/), Cbl is freed in the stomach following proteolytic cleavage of the food stuffs. While still within the stomach, freed Cbl is bound by the protein haptocorrin, which is thought to protect the vitamin against further hydrolysis by the acidic environment.[7,8] Haptocorrin itself is degraded in the duodenum by proteases stemming from the pancreas, following which intrinsic factor (IF) sequesters the released Cbl.[7,8] IF is synthesized and secreted by parietal cells of the stomach, and its deficiency, either due to inborn errors or an autoimmunity against the parietal cells, results in classical pernicious anemia.[9,10] At the terminal ileum, IF-bound Cbl (IF-Cbl) is recognized by distinct cells (polarized epithelial enterocytes), which express a complex receptor composed of a heterodimer of amnionless and cubilin (cubam) in the apical brush border, which mediates cellular absorption of Cbl via endocytosis.[11–13] Malabsorption at this step occurs in the Imerslund-Gräsbeck syndrome, a rare disorder of Cbl deficiency[14,15] caused by mutations of cubilin (*CUBN*)[16] or amnionless (*AMN*).[17]

Inside the cell, the IF-Cbl complex is released from cubam, which recycles to the plasma membrane.[12] IF is degraded by the increasingly acidic environment during the transition from endosome to lysosome. The exact details of Cbl export across enterocytes remains to be clarified, but likely requires many of the same steps as intracellular Cbl processing in unpolarised cells (see next section). Export of free Cbl from the cell into the bloodstream is mediated by at least the basolateral multidrug resistance protein 1 (MRP1),[18] although other methods/transporters may also be involved.[19] In the blood, Cbl may be bound to either of two proteins—haptocorrin or transcobalamin (TC).[20] The far greater proportion is bound by haptocorrin, but the affinity of haptorrin for Cbl is lower than that of TC. Haptocorrin-bound Cbl is not available to most cells. TC-bound Cbl, however, is readily taken up by most cells of the body due to recognition by CD320, the TC receptor.[21] *CD320* is expressed in virtually all tissues,[22] and CD320-mediated uptake promotes TC-Cbl endocytosis. In the kidney, however, an important organ for Cbl storage and recycling,[23] uptake of TC-Cbl is instead mediated by the protein megalin on the apical membrane of proximal tubule cells.[24,25]

3 | INTRACELLULAR COBALAMIN TRANSPORT AND MODIFICATION

Following uptake of TC-Cbl via receptor-mediated endocytosis, intracellular synthesis of the cofactors AdoCbl and MeCbl depends on intracellular transport and modification of the incoming Cbls. The first step of this process is the release of Cbl from TC, which occurs by the same process as that for release of IF in enterocytes, that is, most likely by progressive release from and degradation of the sequestering protein in the acidic environment of the lysosome. Transport of the now free Cbl out of the lysosome and into the cytosol requires two integral membrane proteins, lipocalin-1-interacting membrane receptor domain-containing 1 (LMBD1)[26] and adenosine triphosphate (ATP)-binding cassette subfamily D member 4 (ABCD4).[27] Disturbed function of either of these proteins, which occurs in patients with *cblF* and *cblJ* defects respectively, results in accumulation of Cbl in the lysosome.

After export into the cytosol, Cbl is bound by the protein methylmalonic aciduria cblC type with homocystinuria (MMACHC), which then processes all incoming Cbls to a common cob(II)alamin form. MMACHC has been shown structurally[28,29] to bind Cbl in a special base-off manner, which facilitates biochemical removal of the upper axial ligand either by reductive decyanation[30] or dealkylation.[31] Mutation of *MMACHC*, which occurs in patients with the *cblC* defect,[33] results in decreased availability of Cbl for downstream enzymes, and the inability of cells to metabolize and therefore utilize certain Cbl forms (eg, CNCbl). Following reduction to cob(II)alamin, MMACHC-chaperoned Cbl is then targeted to either MS or MUT by methylmalonic aciduria cblD type with homocystinuria (MMADHC),[34] a protein which interacts with MMACHC only after MMACHC has bound and processed Cbl.[35] MMADHC contains a mitochondrial leader sequence at its N-terminus, and has been identified in both the cytosol and mitochondria.[36] Mutation of *MMADHC*, as in the *cblD* defect,[37] represents the most unique genotype-phenotype relationship in intracellular Cbl processing, whereby: (a) truncating mutations at the N-terminus do not affect delivery of Cbl to MS, but instead result in dysfunction of only MUT; (b) truncating mutations in the middle and toward the C-terminus of the protein result in disruption of cofactor synthesis/delivery to both enzymes; and (c) certain missense mutations near the C-terminus result in dysfunction of MS only.[37–39]

4 | THE MITOCHONDRIAL COBALAMIN PATHWAY

The mechanism by which Cbl enters the mitochondrion remains one of the great mysteries of Cbl metabolism. However, the recent discovery of mutation of wht-6 in *Caenorhabditis elegans* causing disruption of the mitochondrial but not cytosolic Cbl pathway in this organism is a tantalizing clue that a human ortholog in the ATP-binding cassette type G (ABCG) family of transporters may be the long sought after mitochondrial importer.[40] Once inside in the

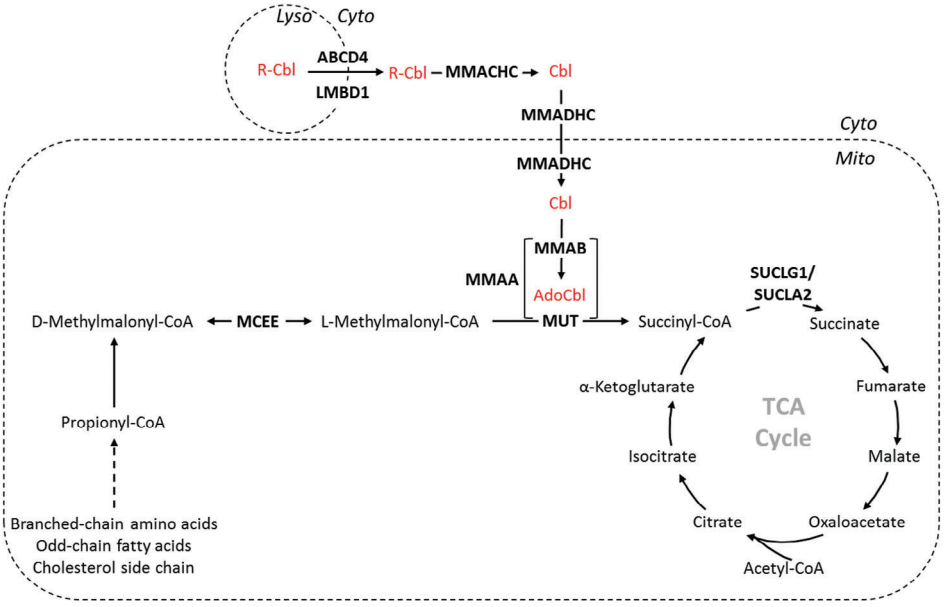

FIGURE 2 Intersection of the methylmalonyl-CoA catabolic pathway with adenosylcobalamin cofactor synthesis. Arrows depict enzymatic reactions. Protein names are in bold. Cobalamin forms are in red. **ABCD4**, ATP-binding cassette subfamily D member 4; **AdoCbl**, adenosylcobalamin; **Cbl**, cobalamin (no upper axial ligand attached); CoA, coenzyme A; **Cyto**, cytosol; **LMBD1**, lipocalin-1-interacting membrane receptor domain-containing 1; **MCEE**, methylmalonyl-CoA epimerase; Mito, mitochondrion; **MMAA**, methylmalonic aciduria cblA type; **MMAB**, methylmalonic aciduria cblB type; **MMACHC**, methylmalonic aciduria cblC type with homocystinuria; **MMADHC**, methylmalonic aciduria cblD type with homocystinuria; **MUT**, methylmalonyl-CoA mutase; **R-Cbl**, cobalamin with upper axial ligand (eg, cyano-, hydroxo-) attached; **SUCLA2**, succinate-CoA ligase ADP-forming beta subunit; **SUCLG1**, succinate-CoA ligase alpha subunit

mitochondria (Figure 2), Cbl may be further chaperoned in conjunction with MMADHC; however, data supporting this, other than the observation that MMADHC is found within the mitochondria, remain lacking. Mitochondrial Cbl is sequestered by the protein methylmalonic aciduria cblB type (MMAB), which catalyzes the ATP-dependent synthesis of AdoCbl,[41] the cofactor form of MUT. Patients with genetic deficiency of *MMAB*, as in the *cblB* defect,[42,44] suffer from isolated methylmalonic aciduria (MMAuria) related to inadequate function of MUT. There is a mutational hotspot in exon 7 of MMAB,[32] which encodes the active site of the protein.[41] By analogy to bacterial homologs, AdoCbl transfer from MMAB to MUT likely proceeds via direct association between the two proteins.[45] This transfer, however, is gated by a third protein methylmalonic aciduria cblA type (MMAA), which additionally appears to protect the AdoCbl from oxidation during the MUT catalytic cycle in a guanosine triphosphate GTP dependent manner.[46,47] Mutation of *MMAA* in patients is described as the *cblA* defect,[43] and every patient mutation described to date either results in a complete loss of MMAA protein or interferes with the interaction between MMAA and MUT.[46]

MUT itself catalyzes the rearrangement of L-methylmalonyl-CoA to succinyl-CoA in an AdoCbl dependent manner. This pathway is an extension of the propionate catabolic pathway, which is required for the breakdown of the branched-chain amino acids valine, isoleucine, methionine and threonine, odd-chain fatty acids and the side chain of cholesterol, and whose product enters the tricarboxylic acid cycle as an anapleurotic substrate. The *mut*-type MMAuria due to mutation of *MUT* is the most common cause of isolated MMAuria.[1] The MUT protein consists of two domains, an N-terminal substrate-binding and a C-terminal cofactor-binding domain connected by a short linker, which exists in a homodimeric state.[48] Many of the patient mutations which cause the generally more severe mut^0 subtype of disease occur within the substrate-binding domain, whereas mutations within the cofactor-binding domain often result in the usually later onset mut^- disease subtype which at least in vitro is Cbl responsive.[49,50] Mutation of *MCEE*,[51] the enzyme directly upstream of MUT in the propionate catabolic pathway, as well as in *SUCLG1*[52] or *SUCLA2*[53,54] the genes encoding the heterodimeric enzyme succinate-CoA ligase immediately downstream of MUT, also result in disease including MMAuria of milder degree.

5 | THE CYTOSOLIC COBALAMIN PATHWAY AND THE METHIONINE (REMETHYLATION) CYCLE

Successful delivery of Cbl to MS within the cytosol depends on the concerted actions of MMACHC and

MMADHC, both of which have been shown to physically interact with MS.[55] Once Cbl has been successfully delivered to MS, it is bound and reduced in such a way that cob(I)alamin is formed. This is the most reduced and reactive Cbl form, which is primed to bind the methyl group from the substrate 5-methyltetrahydrofolate and transfer it to homocysteine, forming methionine and tetrahydrofolate (THF) as products. Loss of MS function, owing to mutation of the encoding gene *MTR*, is defined as the *cblG* defect. This defect, or blocks in the synthesis of the cofactor MeCbl, results in accumulation of homocysteine (hyperhomocysteinemia, homocystinuria). A common dysfunction of the MS enzymatic reaction is the inadvertent oxidation of cob(I)alamin to cob(II)alamin, occurring approximately every 1:200-1000 catalytic turnovers.[56] This cofactor oxidation renders MS enzymatically inactive, but it can be reactivated by methionine synthase reductase (MSR) via reductive methylation, a process which involves transfer of an electron from MSR to MS, coupled with the transfer of a methyl group from AdoMet.[57] Dysfunction of MSR, through mutation of its encoding gene *MTRR*, is defined as the *cblE* defect.[58]

Intracellularly synthesized methionine, along with exogenous sources, is available for incorporation into proteins or further processing by the methionine cycle (Figure 3). The next step in the methionine cycle is the formation of AdoMet from methionine and adenosine triphosphate by methionine adenosyltransferase (MAT). MAT consists of a liver-specific isoenzyme (MAT1A) and a ubiquitously expressed isoenzyme (MAT2A) whose enzymatic activity is regulated by an associated subunit (MAT2B). Dysfunction of MAT1A is genetically linked with an inborn metabolic disorder of hypermethioninemia,[59,60] while MAT2A has been implicated in predisposition to thoracic aortic aneurysms.[61] AdoMet is the principal methyl donor in biological transmethylation reactions, acting as substrate for a plethora of methyltransferases (MTs). An estimated 1% of human genes encode MTs.[62] These enzymes are involved in numerous cellular pathways and are responsible for methylation of metabolites, DNA, RNA, and proteins,[63] including histones.[64] Following transfer of the methyl-group, S-adenosylhomocysteine (AdoHcy) is formed. This reaction proceeds in the forward direction in vivo provided the products are removed. To this end, AdoHcy is processed to homocysteine and adenosine by S-adenosylhomocysteine hydrolase (AHCY). Mutation of *AHCY* is another cause of hypermethioninemia.[65] Successful formation of homocysteine by AHCY completes the methionine cycle, creating a homocysteine molecule that can be remethylated by MS, or combined with serine to form cystathionine by cystathionine beta-synthase as the first step in the transsulfuration pathway. Alternatively, homocysteine may be remethylated by betaine

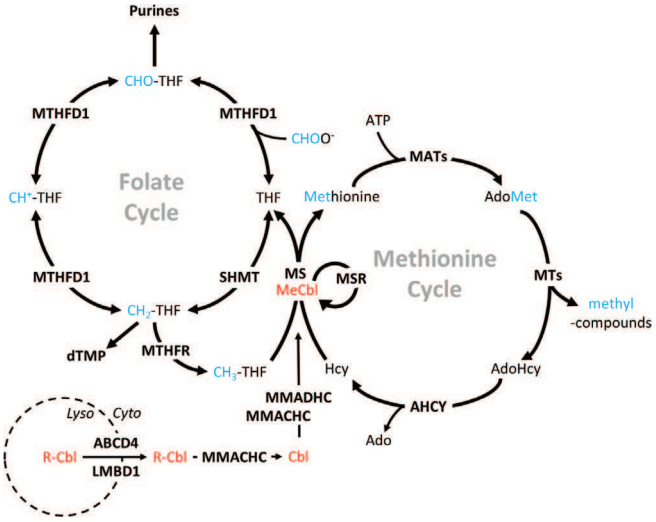

FIGURE 3 The remethylation pathway as depicted through the folate cycle, methionine cycle and intracellular production of MeCbl. Arrows depict enzymatic reactions. Protein names are in bold. Cobalamin forms are in red. Single carbon groups originating from formate and ending on methylated compounds are represented in blue. **ABCD4**, ATP-binding cassette subfamily D member 4; **Ado**, adenosine; **AdoHcy**, adenosylhomocysteine; **AdoMet**, S-adenosylmethionine; **AHCY**, adenosylhomocysteinase; **ATP**, adenosine triphosphate; **Cbl**, cobalamin (no upper axial ligand attached); **CH^+-THF**, 5,10-methenyltetrahydrofolate; **CH_2-THF**, 5,10-methylenetetrahydrofolate; **CH_3-THF**, 5-methyltetrahydrofolate; **CHO-THF**, 10-formyltetrahydrofolate; **$CHOO^-$**, formate; **dTMP**, deoxythymidine monophosphate; **Cyto**, cytosol; **LMBD1**, lipocalin-1-interacting membrane receptor domain-containing 1; **Lyso**, lysosome; **MATs**, methionine adenosyltransferase(s); **MeCbl**, methylcobalamin; **MMACHC**, methylmalonic aciduria cblC type with homocystinuria; **MMADHC**, methylmalonic aciduria cblD type with homocystinuria; **MS**, methionine synthase; **MSR**, methionine synthase reductase; **MTHFD1**, methylenetetrahydrofolate dehydrogenase 1, cyclohydrolase and formyltetrahydrofolate synthetase 1; **MTHFR**, methylenetetrahydrofolate reductase; **MTs**, methyltransferase(s); **R-Cbl**, cobalamin with upper axial ligand (eg, cyano-, hydroxo-) attached; **SHMT**, serine hydroxymethyltransferase

homocysteine methyltransferase, an enzyme that is most strongly expressed in the liver and kidney[66,67] and may play a substantial role in production of methionine in those tissues.

A description of the genes/proteins involved in intracellular Cbl metabolism and the remethylation cycle is provided in Table 1.

6 | FOLATE-MEDIATED ONE-CARBON METABOLISM

The source of the MS substrate 5-methyltetrahydrofolate (CH_3-THF) is the one-carbon metabolic cycle, whose carrier is folate. Folates encompass a set of molecules that contain

TABLE 1 Description of genes/proteins involved in intracellular Cbl metabolism and remethylation

Protein name	Gene name	Complementation group	Protein cellular location	Cellular/enzymatic function
LMBD1	*LMBRD1*	*cblF*	Lysosome	Transport of Cbl across lysosomal membrane?
ABCD4	*ABCD4*	*cblJ*	Lysosome	Transport of Cbl across lysosomal membrane?
MMACHC	*MMACHC*	*cblC*	Cytosol	Cbl chaperone/enzyme
MMADHC	*MMADHC*	*cblD*	Cytosol/Mito	Targeting MMACHC-Cbl complex to MS/MUT?
MMAA	*MMAA*	*cblA*	Mito	Protection of MUT
MMAB	*MMAB*	*cblB*	Mito	ATP + cob(I)alamin → AdoCbl + PPPi
MUT	*MUT*	*mut*	Mito	L-methylmalonyl-CoA → succinyl-CoA
MS	*MTR*	*cblG*	Cytosol	Homocysteine + CH_3-THF → methionine + THF
MSR	*MTRR*	*cblE*	Cytosol	Cob(II)alamin + AdoMet → methylcobalamin + AdoHcy
MTHFD1	*MTHFD1*		Cytosol/nucleus	$CHOO^-$ + THF → CHO-THF → CH^+-THF → CH_2-THF
MTHFR	*MTHFR*		Cytosol	CH_2-THF → CH_3-THF
SHMT	*SHMT1*		Cytosol/nucleus	CH_2-THF + glycine → THF + serine
MATI/III or II	*MAT1A/MAT2A*		Cytosol	ATP + methionine → AdoMet + PPPi
MT	*Various*		Various	Substrate + AdoMet → methyl-substrate + AdoHcy
AHCY	*AHCY*		Cytosol	AdoHcy → adenosine + homocysteine

Mutation of genes above the dashed line results in inherited disorders of cobalamin metabolism, below results in disorders of remethylation/methionine cycle.

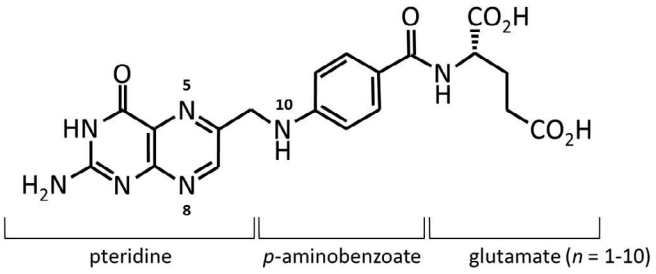

FIGURE 4 Structure of folic acid. Nitrogen-5, −8 and − 10, are acceptors of hydrogen and/or carbon groups. Glutamate may exist as n = 1 (monoglutamate) or as a chain of n = 2-10 (polyglutamate)

(a) a pteridine ring that can be oxidized or reduced; (b) a para-aminobenzoic acid linker that together with the pteridine ring may bind one-carbon units; and (c) a variable-length chain polyglutamate tail (Figure 4). Folic acid, an oxidized folate form and a commonly used synthetic food additive, is reduced at the double bond at nitrogen-8 to produce dihydrofolate (DHF), while further reduction of the double bond at nitrogen-5 generates THF, the active coenzyme form. Both enzymatic steps are catalyzed by dihydrofolate reductase (DHFR). Once reduced to THF, nitrogen-5 and/or nitrogen-10 may serve as acceptors of single carbon units. These carbons may be transferred at varying oxidation states, as determined by their source and the enzyme catalyzing the reaction.

CH_3-THF is the most common folate form in blood and tissues.[68] However, other folate forms in which the bound carbon has a higher oxidation state also have vitally important cellular roles. For example, 5,10-methylene-THF (CH_2-THF) is used by thymidylate synthase (TYMS) for the production of dTMP from deoxyuridine monophosphate (dUMP), an important step in DNA synthesis, while 10-formyl-THF (CHO-THF) is required as a carbon donor for two steps in de novo purine synthesis. The interconversion between different folate forms occurs through the folate-mediated one-carbon metabolic pathway, such that the amount of a particular oxidation state of one-carbon-bound THF produced can be tailored according to cellular needs. This pathway is mainly compartmentalized between the cytosol and mitochondria, with smaller folate amounts found in the nucleus[69] (nuclear folate not discussed here, see for example, [70]). An important determinant of compartmentalization is the polyglutamate tail, which appears to serve as a localization signal within the cell. Monoglutamate but not polyglutamate forms is transported across the intestine by the proton-coupled folate transporter,[71] the plasma membrane by the reduced folate carrier[72] and across the mitochondrial membrane by the mitochondrial folate transporter,[1,73] whereby polyglutamate forms are trapped intracellularly within the compartment the glutamate tail was added. This means circulating folates are also in the monoglutamate form, which may additionally be processed by the folate receptor that has particular significance at the choroid plexus.[74] Another contributor to compartmentalization is that folates with one-carbon units bound (eg, CHO-THF, CH_3-THF), are

not known to cross the mitochondrial membrane.[75] Finally, only a limited number of nonfolate metabolic intermediates within the one-carbon metabolic pathway are able to cross the mitochondrial membrane. Together, these lead to separation of the mitochondrial and cytosolic one-carbon metabolic pathways, which are connected only via specific metabolites. These separated pathways remain functional, however, because a complete set of enzymes exist in both compartments. It is not clear why a parallel set of enzymes in the cytosol/mitochondria are required, especially when the vast majority of one-carbon need is in the cytosol. However, an important clue may come from the finding that mitochondrial one-carbon oxidation accounts for approximately 50% of the NADPH produced in the cell,[76] a massive reducing and energy source. From this has stemmed the hypothesis that one-carbon oxidation is localized to the mitochondria in order to uncouple it from glycolysis, which might be blocked by the depleted NAD^+ levels that would arise should one-carbon oxidation take place in the cytosol.[73]

In humans, the major source of one-carbon units is serine, which in the presence of THF is reversibly metabolised to glycine and CH_2-THF by the enzyme serine hydroxymethyltransferase (SHMT). At least two different isozymes of SHMT (cytosolic SHMT1 and mitochondrial SHMT2) exist, encoded by separate genes (*SHMT1* and *SHMT2*, respectively). Studies using deuterated serine have demonstrated that the majority of one-carbon units transferred to methionine in healthy volunteers[77] and in cell culture[78] originate from the mitochondria (ie, starting from SHMT2), and this has been found to be the case in most cancer cell types as well.[79] Therefore, the folate cycle may be thought to begin with demethylation of serine by mitochondrial SHMT2 in the presence of free THF, to form glycine and CH_2-THF (Figure 5). The cycle continues with the two-step oxidation of CH_2-THF to methenyl-THF (CH^+-THF) and then CHO-THF by bifunctional methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) or MTHFD2 like (MTHFD2L), isoenzymes with different expression patterns.[80,81] The final mitochondrial step is the formation of formate and free THF by methylenetetrahydrofolate dehydrogenase 1 like (MTHFD1L). Formate is an important intermediate that is able to cross the mitochondrial membrane, and whose availability in the cytosol is an important determinant of the direction in which the cytosolic pathway proceeds.[82] Within the cytosolic pathway, methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) catalyzes the incorporation of formate back into free THF to form CHO-THF, a required substrate for de novo purine synthesis, donating two carbon groups to the purine ring through the purinosome enzymes GART and ATIC, respectively (Figures 3 and 5). CHO-THF may alternatively be successively reduced to CH_2-THF by the second and third functions of the trifunctional enzyme MTHFD1. CH_2-THF has alternative fates depending on the needs of the cell: the remethylation of glycine to serine by SHMT1 to complete the folate cycle; the production of dTMP from dUMP for DNA synthesis by TYMS; or final reduction to CH_3-THF by methylenetetrahydrofolate reductase (MTHFR), providing the substrate for MS and the methionine cycle. This last option commits THF for use by MS, as the MTHFR catalyzed reduction is physiologically irreversible and MS is the only enzyme that utilizes CH_3-THF. One important consequence of this is that in the presence of MS deficiency, cellular folates may become trapped in the CH_3-THF form. This results in an inability to

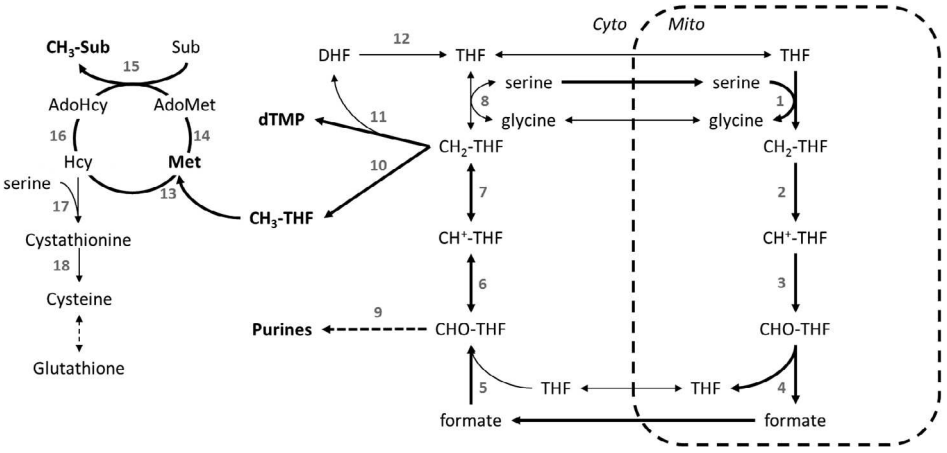

FIGURE 5 Simplified folate-mediated one-carbon metabolism pathway in the cytosol and mitochondria. Arrows represent enzymatic reactions or transmembrane transport. Broken arrows represent multiple enzymatic steps. Numbers indicate the enzyme(s) responsible for the enzymatic reaction as follows (provided where possible as the human gene name): 1) SHMT2, 2) MTHFD2/MTHFD2L, 3) MTHFD2/MTHFD2L, 4) MTHFD1L, 5) MTHFD1 (synthetase), 6) MTHFD1 (cyclohydrolase), 7) MTHFD1 (dehydrogenase), 8) SHMT1, 9) GART and ATIC (in the de novo purine synthesis pathway), 10) MTHFR, 11) TYMS, 12) DHFR, 13) MTR, 14) MAT1A or MAT2A, 15) AdoMet-dependent methyltransferase, 16) AHCY, 17) CBS, 18) CTH

produce purines and dTMP, which is especially debilitating for quickly dividing cells (eg, blood cells in the bone marrow) and is the presumed cause of megaloblastic anaemia in Cbl deficiency.

The relative importance of each endpoint in the cytosolic pathway (purine synthesis, dTMP synthesis or methionine/AdoMet synthesis) has been investigated by supplying labeled serine or formate to various cell types, followed by determination of the relative incorporation of label to each of these final compounds. In quickly proliferating cells, including cultured cancer cells, stem cells, hematopoietic cells, and during development, the majority of labeled carbons are incorporated into purines and dTMP (reviewed in:[73,83]), reflecting the predominant requirement of these cell types for DNA synthesis. In these situations, cells are exquisitely sensitive to folate depletion, manifesting in the efficacy of the folate analogs methotrexate and pemetrexed for cancer treatment,[84] in neural tube defects arising from folate insufficiency[85] or mouse knockouts of mitochondrial folate processing enzymes,[86] and in the macrocytic/megaloblastic anaemia presented in patients with insufficient folate uptake or blockages in folate processing.[1] In slowly proliferating fibroblasts in culture, Stover and colleagues[87] found that compared to control cells, the incorporation of formate into methionine of MTHFD1 deficient fibroblasts was decreased by 90% and into dTMP by 50%, but purine incorporation was unchanged. This again is consistent with sacrifice of the methionine cycle in favour of protecting nucleotide synthesis in this cell type. In nonproliferating cells, however, cellular requirements are different. In the adult liver and kidney, two major reservoirs of anabolic one-carbon reactions, a significant proportion of one-carbon groups likely flow through the methionine cycle. In these tissues, methionine cycle produced AdoMet is required for the synthesis of creatine and phosphotidylcholine, creation of which account for up to 80% of all methylation reactions in the body.[88]

7 | ALLOSTERIC, GENETIC, AND EPIGENETIC REGULATION

Due to the vital nature and interconnection of the intracellular Cbl, methionine synthesis and one-carbon metabolic pathways, these processes undergo regulation to ensure the best use of the often limited substrates or cofactors available. While some of these regulation mechanisms have been known for some time, for example, allosteric enzyme modulation by AdoMet and AdoHcy, exciting emerging concepts regarding regulation at the gene, mRNA and enzyme level have recently been identified.

In terms of intracellular Cbl cofactor synthesis, there is now compelling evidence for the regulation of *MMACHC* expression. The first piece of evidence was provided in patients who had loss of MMACHC activity and expression despite harbouring no identifiable mutations in the *MMACHC* gene. These patients were eventually found to harbour mutations in the kelch domain of the transcriptional coregulator host cell factor C1 (HCFC1),[89–91] a molecular scaffold whose function is critical for cell proliferation and cell-cycle progression.[92,93] Control of the cell cycle by HCFC1 has been shown to require formation of a complex with the transcription factors THAP domain-containing protein 11 (THAP11) and zinc finger protein 143 (ZNF143).[94] Indeed, mutation of both THAP11[95] and ZNF143[96] has been shown to result in loss of *MMACHC* expression and clinical Cbl deficiency, thus demonstrating that this complex is also involved in *MMACHC* expression. This provides a link between Cbl cofactor synthesis and cell growth and proliferation, which connects the methionine and one-carbon cycles as well (see below). Interestingly, putative HCFC1 binding sites were also identified in the promoters of *MTR* and *ABCD4*; however, analysis of RNA and protein expression of these genes in fibroblasts showed no difference between HCFC1 patients and controls.[91] In an alternative mechanism, loss of *MMACHC* expression has been described to be due to mutation of its neighbouring antisense oriented gene *PRDX1*, which causes hypermethylation of the promoter and first exon of *MMACHC* and thereby causes *MMACHC* silencing.[97] Thus far, no other regulatory mechanisms have been described for proteins proximal to MMACHC in the intracellular Cbl pathway (ie, LMBD1 and ABCD4) or in the mitochondrial pathway (ie, MMAA, MMAB, MMADHC, and MUT).

MS sits at the conjunction of the Cbl cofactor synthesis pathway and the methionine cycle and therefore may represent a prime location for the regulation of both cycles simultaneously. A potential site of regulation was found on a 70-bp stretch of the 5′-untranslated region *MTR* mRNA. This site was suggested to be modulated by Cbl at an internal ribosome entry site (IRES) in conjunction with an unidentified protein.[98,99] Unfortunately, these tantalizing initial findings have not been followed up, and evidence for the existence of the IRES is disputed.[100] Nevertheless, clues to the presence of regulated expression of *MTR* exist. For example, homocysteine has been found to increase mRNA levels in Caco-2 cells,[101] a link between *MTR* expression and the cell cycle has been found, whereby the *MTR* mRNA level is elevated during the S phase of synchronized U2OS cells,[102] and MS activity is found to be lowest during G_0 and G_1 in human lymphocytes.[103]

Beyond MS, but within the methionine (re)methylation cycle, regulation of pathway activity at the enzymatic level by the metabolites AdoMet and AdoHcy is historically well known. Increased concentrations of AdoMet result in allosteric inhibition of MTHFR[104] and MAT2A[105,106] as well as concomitant activation of cystathionine β-synthase,[107]

ensuring no new AdoMet is produced while excess homocysteine is released from the cycle. By contrast, increased local concentrations of AdoHcy enable dis-inhibition of MTHFR[108] along with inhibition of AdoMet-dependent MTs.[109,110]

Recently, a new intracellular sensor of AdoMet has been described. This new sensor, SAMTOR, has a relatively low dissociation constant for AdoMet of ~7 μM and interacts with the Gap Activity TOward Rags 1 (GATOR1)-complex associated with mammalian Target Of Rapamycin Complex 1 (mTORC1), the major regulator of cell growth and metabolism based on environmental cues,[111] when intracellular AdoMet is depleted. Since methionine and AdoMet production are closely related, SAMTOR has also been suggested to serve as the mTORC1 linked sensor of methionine starvation. A further link between the methionine cycle and mTORC1 has been identified in mice with knockout of nitrogen permease regulator-like 2 (NPRL2), a protein within the GATOR1 complex.[112] NPRL2 knockout mice exhibit low methionine and high CH_3-THF levels characteristic of MS deficiency, which in this case is due to the inability to generate the low lysosomal pH required for release of free Cbl from transcobalamin. This dysfunction ultimately stems from lysosomal gene expression inhibition by the constitutively active mTORC1, and exhibits a mechanism by which mTORC1 may act back on the methionine cycle. Finally, Manning and colleagues[113] found that mTORC1 stimulates de novo purine synthesis in mouse embryonic fibroblasts by inducing *MTHFD2* transcription via activating transcription factor 4 (ATF4), a transcription factor involved in control of the cell cycle.

This latter finding illustrates the connection between these pathways and the cell cycle, which has been found for other proteins as well. A recent review by Stover and colleagues[70] points out that *MTHFD1* is predominantly expressed in the G_1/S and G_2 phases of the cell cycle in human fibroblasts,[114] although the mechanism by which this regulation takes place is unclear. SHMT1 protein levels, by contrast, are elevated during the S phase of HeLa cells, but without changes in mRNA levels.[115,116] Meanwhile, we found that phosphorylation of MTHFR leads to an increased sensitivity to AdoMet inhibition,[117] and the kinase responsible for this phosphorylation has been suggested to be the cyclin dependent kinase 1 (CDK1) in association with cyclin B1.[118] Together, these data provide a link between the cell-cycle state and regulation of proteins involved in methionine cycle or the one-carbon metabolic pathway.

A further level of complexity of regulation exists in that these pathways may in turn regulate the expression of other genes and pathways in the cell. Studies performed mainly in cancer cells have indicated that altered methionine metabolism has an effect on histone methylation via altered function of histone MTs.[119,120] Histone methylation drives gene expression up or down, depending on the modification placement. Since various histone MTs have differing affinities for AdoMet, decreased or fluctuating intracellular concentrations may have a larger impact on some histone modifications than others.[64] In relation to just one type of histone methylation, transcriptionally activating H3K4me3, Locasale and colleagues[121] found that modulating dietary methionine intake was sufficient to alter levels of histone methylation, with rapid changes in H3K4me3 levels and altered gene transcription following changes in methionine availability. Therefore, alterations in the methionine cycle, for example, due to genetic blocks in Cbl or folate pathway proteins, do not just result in hyper- or hypo-methylation, but altered methylation patterns. This is consistent with findings from an epigenome-wide association study performed in 90 mouse inbred strains, which found that genetic variation in *Mtrr* (encoding MSR) affected methylation of almost 500 loci throughout the genome.[122] These methylation changes may have long-term effects, as transgenerational epigenetic inheritance ultimately coming from the maternal grandparents was found to result in intrauterine growth restriction, developmental delay, and congenital malformations in hypomorphic *Mtrr* mice.[123]

In sum, the Cbl, methionine, and folate biochemical pathways are interlinked with other crucial pathways in the cell, with each exerting regulatory influence on the other.

8 | CONCLUSIONS/OUTLOOK

Within the cell, metabolism of Cbl and folate is interrelated. These pathways are joined metabolically at the methionine cycle, but are connected by regulation through other cellular metabolic and regulatory pathways at many points. Although shared function and regulation through particular metabolites have been known for some time, only now are we beginning to realise the extent to which these pathways are interdependent. This continued understanding of the effect alterations in single steps, either via regulation or through genetic blocks, has on all aspects of these pathways may finally bring us to a state where we can better comprehend, and perhaps predict, what will happen during therapeutic modulation or disease. This will be key to better modulating these pathways in health and disease.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

D.S.F. conceived, designed, and drafted the article. B.F. and M.R.B. drafted and provided critical revision of the article. D.S.F. is the guarantor and corresponding author. All authors have read and approved the final version of the manuscript to be published.

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